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Agency

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Laboratory
Cincinnati OH 45268

Research and Development

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EPA

Project Summary

Assessment and Implications of Bacterial Regrowth in Water Distribution Systems

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Betty H. Olson

The objective of this investigation was to establish the extent and nature of bacterial regrowth in water distribution systems. Two water systems were studied: an untreated, unchlorinated groundwater source and a fully treated, chlorinated surface water source. Bacteria were enumerated using six techniques and three media at both source and site (consumer) locations. Over the 2-year study period, 26 chemical parameters were measured bimonthly, quarterly, or semi-quarterly.

Degradation of water bacterial quality was observed in both systems as the water moved from the source to the site location. Higher bacterial counts, turbidity levels, and particle counts were observed at site locations, and bacterial counts were generally greater in the unchlorinated system. Chlorine resistance studies showed that bacteria from the chlorinated supply survived exposure to higher concentrations of chlorine.

Highest bacterial counts were achieved with the membrane filter procedure using m-standard plate count agar (m-SPC), and the second highest counts were produced by low-nutrient medium (R-2A) spread plates. The *Standard Methods* pour plate method recovered the lowest number of bacteria of the six techniques employed.

Bacterial colonization of pipe surfaces was demonstrated in galvanized iron pipe that had been in use for 40 years. Scanning electron microscopy (SEM) revealed bateria such as *Gallionella, Actinomyces, and Prosthecomicrobium* adhering to the interior pipe surface. The elemental composition of the pipe material was examined using energy dispersive x-ray analysis. These analyses indicated that the composition of the deposited pipe material differed dramatically in minor constituents over very small distances (1 mm). This factor may be more important to bacterial colonization of pipe surfaces than the chemical water quality parameters typically measured. A diverse group of bacterial genera were present in both the unchlorinated and chlorinated systems. Four bacterial genera, *Pseudomonas/Alcaligenes, Acinetobacter, Flavobacterium,* and *Klebsiella,* were found at the unchlorinated source; *Acinetobacter, Flavobacterium,* and *Pseudomonas/Alcaligenes* were found at the site location in the unchlorinated system. Except for *Klebsiella,* the same genera were found in the chlorinated system.

This Project Summary was developed by EPA's Municipal Environmental Research Laboratory, Cincinnati, OH, to announce key findings of the research project that is fully documented in a separate report of the same title (see Project Report ordering information at back).

Background

A 2-year investigation was conducted to determine the extent and nature of bacterial regrowth in two California distribution systems receiving different source waters — the Irvine Ranch Water District (IRWD) and the city of

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Garden Grove. The IRWD receives surface water from the Colorado River and from Northern California Project waters. These two sources are blended (60:40, respectively) to achieve a total dissolved solids (TDS) measurement of 500 mg/l. The blended water receives full treatment, consisting of coagulation, flocculation, sedimentation, filtration, and chlorination. The water is chlorinated to maintain a residual of 1.0 mg/l; it is then pumped approximately 33 km to the city of Irvine, where it enters the IRWD system. This system supplies approximately 75,000 persons through 372 km of water mains. The oldest sections of pipe in this system were installed in 1962. Ninety-five percent of the pipe is asbestos-cement, and 5% is plastic-lined steel.

The Garden Grove, California, system is supplied with groundwater obtained from 30 wells in the area. The groundwater is stored in seven reservoirs (four of which are below ground) with a collective storage capacity of 170 million liters (45 million gallons). During the summer months, supplemental water is purchased from the Metropolitan Water District of Southern California to accommodate peak use. Since 1979, supplemental water has accounted for approximately 10% of the total water used. Normally, Garden Grove water is not treated or chlorinated before distribution. But free-chlorine residuals of 0.1 mg/l were detected in some areas of the city when imported chlorinated water entered the system. The Garden Grove system serves 150,000 persons through 607 km of water mains. Pipe materials in this system are asbestos-cement and cast-iron, which is present in a few of the older areas of the city (less than 5% of the total). The oldest sections of pipe were installed in the 1920's.

Methods

Six different techniques were used to enumerate heterotrophic bacteria in the water distribution systems studied. These experiments (Figure 1) indicated that membrane filtration with incubation at 35°C for 4 days on m-SPC medium was the most effective method. The *Standard Methods* pour plate procedure yielded the poorest results.

Results

Bacteria in Water from Source and Site Locations

Approximately 2,000 organisms were identified from two water distribution systems at quarterly intervals from September 1978 to April 1980. Great variation existed in the types of organisms found in the source water and in water taken from site locations (Figures 2 and 3). The composition of these populations was highly variable, with no particular trends being evident over the study period. But predominant genera were present at both the site and the source locations over the study period. At the unchlorinated site and at the chlorinated source and site, predominant genera were *Acinetobacter, Pseudomonas/Alcaligenes,* and *Flavobacterium;* at the unchlorinated source, they included *Acinetobacter, Pseudomonas,* and *Klebsiella.* Isolation of *Klebsiella* from plate count plates did not necessarily correspond with positive total coliform results. The random distribution and low frequency of occurrence of *Klebsiella* and other coliforms affected their isolation from plate count plates. A comparison of bacteria isolated on the different media used (Figure 1) showed that the types of bacteria isolated varied with the medium used.

As the chlorine residual increased, the ratio of pigmented bacteria to total bacteria also increased up to a free residual chlorine level of 0.7 mg/l. Samples with chlorine residuals in excess of 0.7 mg/l did not demonstrate this trend.

Bacterial Colonization within Distribution Lines

Surfaces within water distribution mains and of suspended particulate matter filtered from drinking water were examined using scanning electron microscopy (SEM) to investigate the nature and extent of their microbial association. In addition, x-ray energy dispersive microanalysis (x-ray EDM) was employed to determine the elemental constituents of the pipe surface. In the chlorinated distribution system, a variety of morphologically distinguishable bacterial structures and microcolonies were observed, although they were sparsely distributed along the pipe surface. The mophology of individual cells varied from chain-forming cocci to filamentous and prosthecate-type cells. The iron-oxidizing bacterium, *Gallionella,* recognized by its characteristic helical stalk, was observed both in water samples and attached to iron pipe

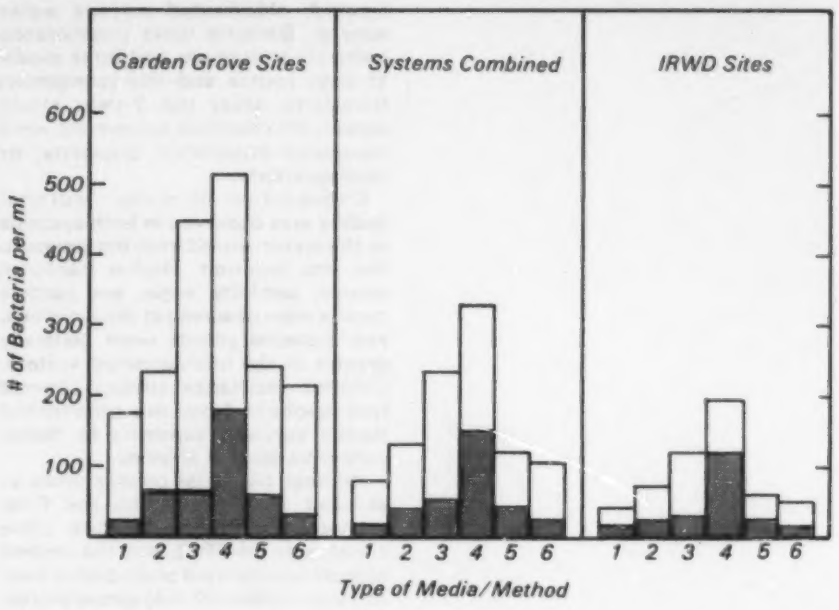

Figure 1. Geometric means of total and pigmented bacterial counts from the Garden Grove system, the Irvine Ranch system, and the combined systems. Key: 1 = plate count agar, pour plate, 35°C; 2 = plate count agar, spread plate, 35°C; 3 = R-2A low nutrient medium; 4 = m-SPC medium; 5 = place count agar, spread plate, 20°C; 6 = plate count agar pour plate, 20°C.

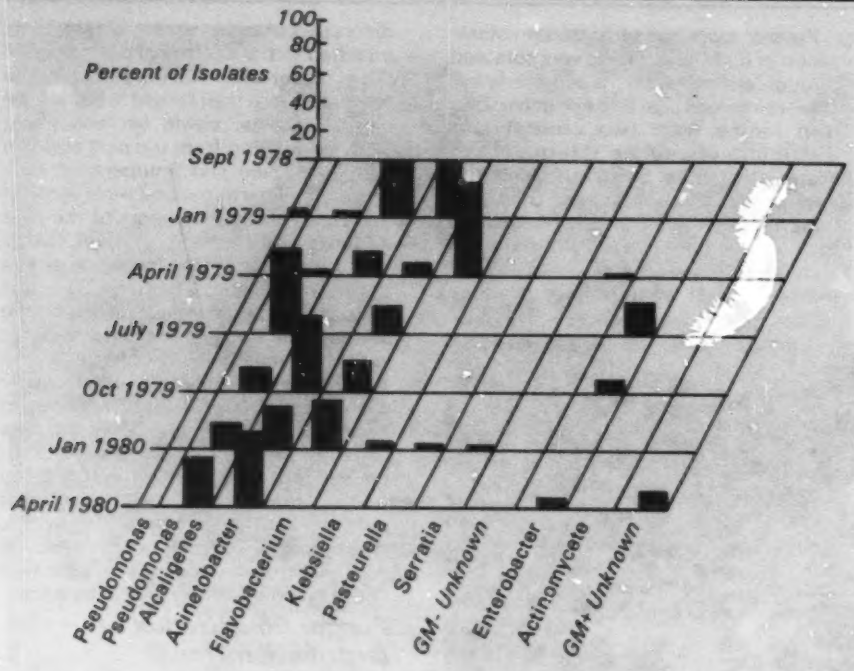

Figure 2. *Bacterial genera represented at Irvine source at the designated sampling intervals.*

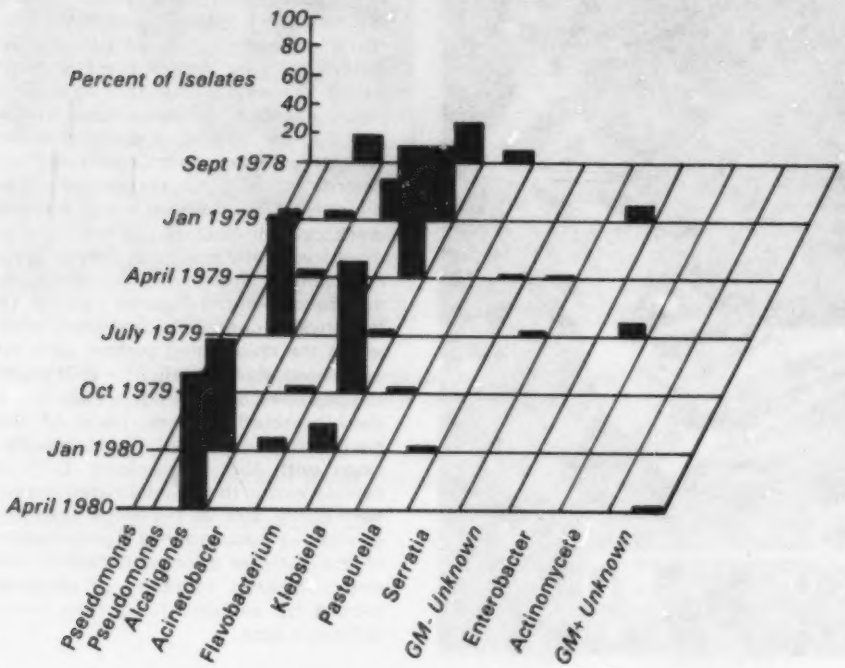

Figure 3. *Bacterial genera represented at Irvine site at the designated sampling intervals.*

surfaces (Figure 4). Attachment of some microbes to pipe surfaces was apparently mediated by extracellular fibrillar appendages; large numbers of rod-shaped bacteria were also evident, adhering to pipe surfaces and on suspended particles recovered from water samples by membrane filtration. Scans of pipe surfaces using x-ray EDM revealed the presence of five major elemental constituents, including silicon, phosphorus, sulfur, calcium, and iron. Smaller quantities of zinc, magnesium, aluminum, potassium, and manganese were also detected.

Iron bacteria belonging to the genus *Gallionella* were also observed by SEM in water samples and attached to galvanized pipe surfaces in the unchlorinated groundwater-supplied drinking water distribution system. The cells were recognized by their characteristic elongated helical stalks composed of numerous intertwined microfibrils. Many of the stalks were partially coated with insoluble ferric salt deposits. Stalks recovered directly from water samples (Figure 5) were analyzed for their elemental composition by using x-ray EDM. Silicon, aluminum, calcium, and iron were the predominant elements

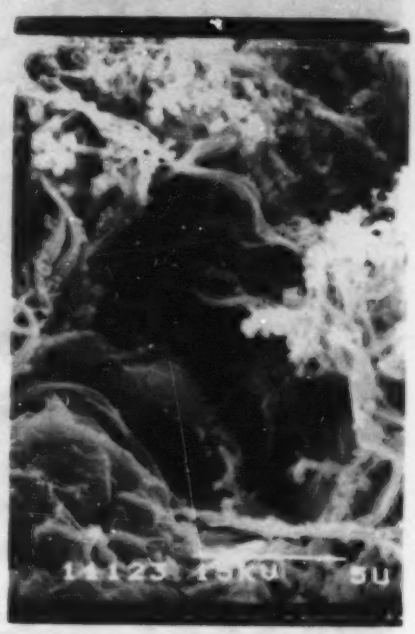

Figure 4. Gallionella *stalks attached to cast iron pipe surface. Note typical striated helices of individual* Gallionella *stalks. Bar = 5 μm.*

present in the stalks. Smaller quantities of phosphorus, sulfur, chlorine, copper, and zinc were also detected. Manganese, although present in measurable quantities in the water supply, was not detected in the stalks, suggesting that this organism was unable to use this element as an electron donor. This work represents the first such analysis of *Gallionella* stalks recovered from environmental samples without prior subculturing in artificial laboratory media.

Further evidence of bacterial colonization of distribution lines was obtained through examination of a cement-lined, steel-reinforced pipe and two galvanized iron mains from two other water distribution systems (Metropolitan Water District of Southern California and Santa Monica Water System). Results from SEM and conventional bacterial isolation and enumeration techniques (Figure 6 and Tables 1 and 2) indicated that bacteria occurred as discrete colonies or as single cells attached to the surface of both types of pipe materials. Although chlorine residuals were maintained in both of the above systems, viable bacteria (most likely originating from the pipe surface) were identified and enumerated. Furthermore, determinations were made of the elemental constituents of the pipe surfaces and of the particulate matter that arises from the surfaces and enters the water supplies. The occurrence of a wide range of inorganic elements on the surfaces of these three pipe samples was observed. Seven different bacterial genera or groups were isolated from the galvanized iron pipe that had been in service for approximately 60 years. The cement-lined pipe showed concentrations of organisms as high as 10^9 colony forming units (CFU)/cm^2 of scraped surface material. Variation in the number of CFU/cm^2 probably resulted because the surface of the pipe was coated with a coal-tar-like substance.

Figure 5. Gallionella *stalk recovered in water samples at a site location supplied with groundwater. Note individual microfibrils that compose stalks.*

Fungal Colonization of Distribution Lines

The frequency of filamentous fungal colonies and physico-chemical and bacteriological parameters were assessed eight times over a 1-year period in the chlorinated IRWD system and the unchlorinated Garden Grove system. Numbers of filamentous fungi were determined by membrane filtration using Czapek-Cox (Difco*) agar. The mean number of filamentous fungal colonies per 100 ml of drinking water was 18 and 34 in the unchlorinated and chlorinated systems, respectively. The majority of filamentous fungi isolated were saprophytic *Deuteromycotina*. The most frequently occurring genera were *Penicillium, Sporocybe, Acremonium,* and *Paecillomyces* (Figures 7 and 8). Of the physico-chemical parameters studied in the chlorinated system, only pH correlated significantly (R = 0.61) with the observed fungal frequencies; in the unchlorinated system, none of the parameters exhibited single correlations with fungal numbers. Lack of correlations in the unchlorinated system was partly due to the large standard deviations encountered. Continuation of the study for a longer period of time and increased numbers of samples would be needed to obtain more definitive data.

*Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

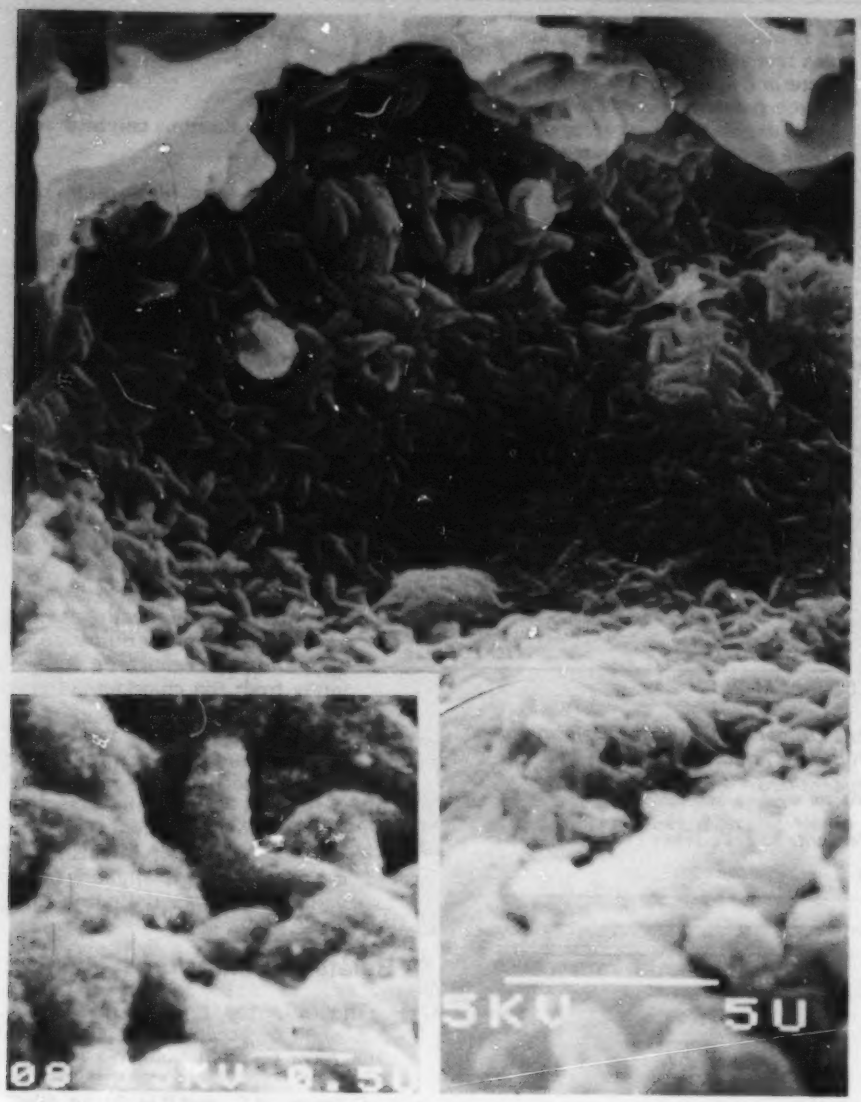

Figure 6. *Scanning electron micrograph of a concave microcolony on a mortar-lined pipe. An enlargement of this colony is shown in the inset.*

Table 1. *Bacterial isolates from the organic surface layer of a mortar-lined pipe of the Metropolitan Water District of Southern California*

Colony forming units/ml	Site no.	% of population	Bacterial genera
10^7	1	10.0	Escherichia coli
		90.0	Acinetobacter *sp.*
10^6	2	50.0	Escherichia coli
		50.0	Serratia *sp.*
10^9	3	100.0	Acinetobacter *sp.*
10^8	4	100.0	Acinetobacter *sp.*

Effect of Bacteria and Fungi on Traditional Test Methods

The occurrence of both bacteria and fungi in water distribution systems raises the question of what effects these organisms might have on traditional testing techniques such as the coliform test. To determine whether the bacterial isolates from the unchlorinated groundwater supply and the chlorinated surface water supply were having an inhibitory effect on coliforms, the isolates were screened by the agar overlay method for the ability to produce bacteriocin-like substances (BLS) inhibitory to growth of *Escherichia coli*, *Klebsiella* sp., and *Enterobacter aerogenes*. Production of coliform-specific BLS by noncoliform bacteria varied with site and date of isolation as well as the genus of the producer bacterium (Tables 3 through 6). A total of 448 bacterial isolates from the chlorinated system were screened, and 22.1% produced BLS specific for at least one of the three coliforms. In the unchlorinated system, 7.9% (n = 696) of the isolates screened produced by BLS. *Flavobacterium/Moraxella* constituted 57.1% of all bacteria (from both systems) producing BLS. The possibility that BLS interferes with coliform detection in standard bacterial quality tests appears to have some merit based on observations with the most-probable-number (MPN) method and with membrane filtration methods.

Chlorine Sensitivity of Bacterial Isolates

Finally, two different methods were used to examine the relative chlorine sensitivity of bacteria isolated from chlorinated and unchlorinated drinking water distribution systems. One method measured the toxic effect of free chlorine on the bacteria, and the other measured the effect of combined chlorine. The effect of combined chlorine is important to understand because many water treatment systems throughout the United States are considering the use of a combined chlorine residual (chloramines) to reduce occurrence or production of trihalomethanes (THM's) as a result of the chlorination procedure. Bacteria isolated from the chlorinated system were more resistant to both the combined and free forms of chlorine than were the bacteria isolated from the unchlorinated system. These results suggest that selection for chlorine-tolerant microorganisms may occur in chlorinated distribution systems.

Bacteria on particulates retained on the surface of 2.0-μm Nuclepore membrane filters were significantly more resistant to free chlorine compared with the total microbial population recovered on 0.2-μm membrane filters. The reason is presumably that aggregated cells or bacteria attached to suspended particulate matter exhibit more resistance than unassociated microorganisms. This hypothesis was confirmed when SEM examination of suspended particulate matter from the water samples revealed the presence of attached bacteria (Figure 9). The more resistant microorganisms were able to survive a 2-min exposure to 10 mg free chlorine/l. These included gram-positive spore-forming bacilli, *Actinomycetes*, and micrococci. The more sensitive bacteria were readily killed by chlorine concentrations of 1.0 mg/l or less; these included gram-positive micrococci, *Corynebacterium*, *Pseudomonas/Alcaligenes*, *Flavobacterium/Moraxella*, *Klebsiella*, and *Acinetobacter*.

Table 2. Bacterial isolates recovered from galvanized iron pipe from the Santa Monica, California, water system

	% of total isolated*	
Bacterial genera	*SM-1*	*SM-2*
Pseudomonas/Alcaligenes	47.8	3.4
Serratia	4.4	0.0
Flavobacterium/Moraxella	4.4	1.7
Acinetobacter	0.0	5.2
Bacillus	13.0	1.7
Corynebacterium/Arthrobacter	25.1	75.9
Caulobacter	0.0	5.2
Unknown	4.4	6.9
Total	100.0	100.0

**Total number isolated is 23 for SM-1 and 58 for SM-2.*

Mean Percentage Frequencies of Filamentous Fungal Genera in the Chlorinated Drinking Water Distribution System
(Mycelia sterila = 2.8%, Unidentified = 4.6%)

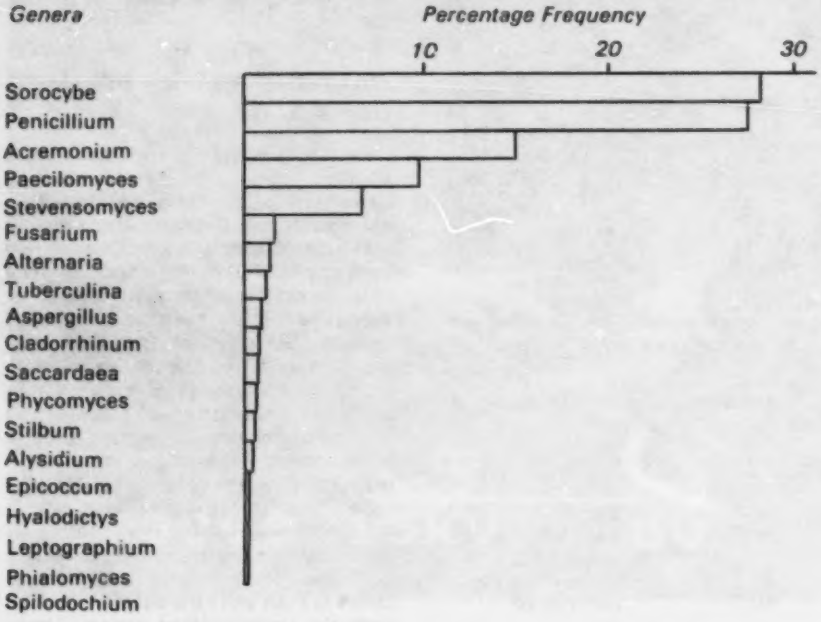

Figure 7. Mean percentage frequencies of filamentous fungal genera in the chlorinated drinking water distribution system. (Mycelia sterila = 2.8%; unidentified = 4.6%).

Conclusions

Several generalizatons can be drawn from this study.

- The membrane filter technique using m-standard plate count (m-SPC) and low nutrient R-2A medium spreud plates with 96-hr incubation periods yielded the highest total plate counts. The pour plate technique yielded the lowest counts.
- Different media appeared to recover different bacterial populations, as shown by pigmented counts obtained on various media tested.
- SEM indicated that bacterial microcolonies as well as single bacterial cells were associated with galvanized pipe sections and free floating particles from the Garden Grove Distribution system.
- Residual chlorine levels showed negative correlation with bacterial counts, but small numbers of bacteria were recovered consistently from the chlorinated Irvine system.
- Bacteria isolated from the treated Irvine system were more resistant to combined chlorine than were those isolated from the untreated Garden Grove system.
- Chemical parameters measured did not provide explanations of the variations in bacterial numbers or types.

Related Publications

The following is a list of articles, published or accepted for publication, that have resulted from this research project.

Ridgway, H.F., and B.H. Olson. 1981. Scanning Electron Microscope Evidence for Bacterial Colonization of a Drinking-Water Distribution System. Appl. Environ. Microbiol. 41(1):274-287.

Ridgway, H.F., E.G. Means, and B.H. Olson. 1981. Iron Bacteria in Drinking-Water Distribution Systems: Elemental Analysis of *Gallionella* Stalks, Using X-Ray Energy-Dispersive Microanalysis. Appl. Environ. Microbiol. 41(1):288-297.

Olson, B.H., and L. Hanami. 1981. Seasonal Variation of Bacterial Populations in Water Distribution Systems. Technology Conference Proceedings, Advances in Laboratory Techniques for Quality Control,

Mean Percentage Frequencies of Filamentous Fungal Genera in the Unchlorinated Drinking Water Distribution System

(Mycelia sterila = 2.5%, Unidentified = 5.7%)

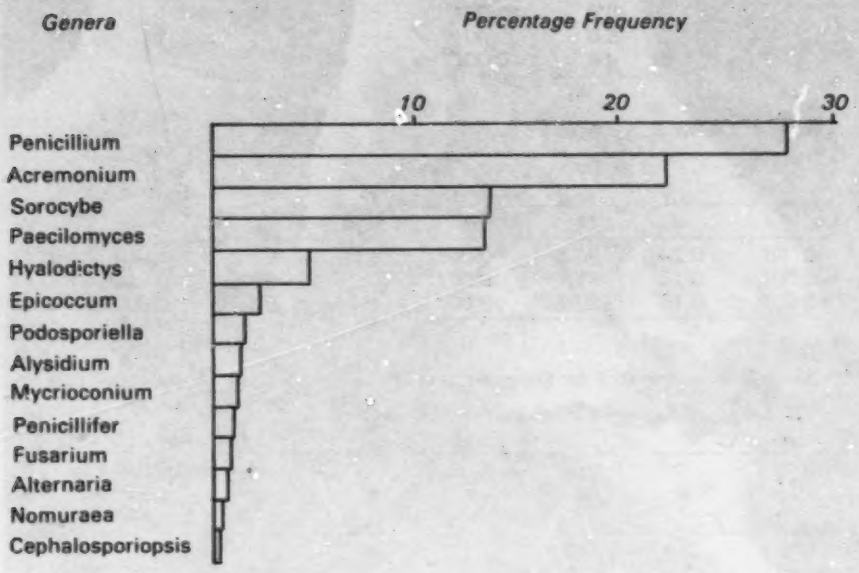

Figure 8. *Mean percentage frequencies of filamentous fungal genera in the unchlorinated drinking water distribution system. (Mycelia sterila = 2.5%; unidentified = 5.7%).*

p. 137-151. American Water Works Association, Denver, Colorado.

Means, E.G., L. Hanami, H.F. Ridgway, and B.H. Olson. 1981. Enumeration of Bacteria in Potable Water Distribution Systems: Evaluation of Media and Plating Techniques. Jour. Amer. Water Works Assoc., 73(11):585-590.

Means, E.G., and B.H. Olson. 1981. Coliform Inhibition by Bacteriocin-like Substances in Drinking Water Distribution Systems. Appl. Environ. Microbiol. 42(3):506-512.

Ridgway, H.F., and B.H. Olson. 1982. Chlorine Resistance Patterns of Bacteria from Two Drinking-Water Distribution Systems. Appl. Environ. Microbiol. (Accepted for publication).

Nagy, L.A., and B.H. Olson. 1982. The Occurrence of Filamentous Fungi in Drinking Water Distribution Systems. Can. Jour. Microbiol. (Accepted for publication).

Olson, B.H., H.F. Ridgway, E.G. Means, and D.L. Johnson. 1982. Bacterially Colonized Particles in Potable Ground Water: Occurrence and Chemistry. Jour. Environ. Sci. Tech. (Accepted for publication).

The full report was submitted in fulfillment of Grant No. R805680010 by the University of California, Irvine, CA, under the sponsorship of the U.S. Environmental Protection Agency.

Table 3. *Effect of sample location and sampling time on the bacterial population producing BLS*

Site or date of isolation	No. tested	% of isolates producing coliform-inhibitory substance*	Isolates producing inhibitor specific for					
			E. coli		*Klebsiella* sp.		*E. areogenes*	
			No.	%	No.	%	No.	%
Site								
Irvine Source	196	5.6	3	1.5	3	1.5	5	2.6
Paseo Picasso	252	34.9	55	21.8	40	15.9	25	9.9
Well 6064	129	4.6	4	3.1	3	2.3	0	0.0
Well 6321	196	14.3	15	7.5	12	6.0	8	4.0
Well 23/9	34	2.9	1	2.9	8	0.0	0	0.0
Rockinghorse	337	5.9	9	2.7	8	2.4	7	2.1
Date								
2 Jan. 1979	126	8.7	4	3.2	4	3.2	6	4.8
25 Jan. 1979	175	14.8	15	8.6	8	4.6	7	4.0
26 Apr. 1979	148	8.5	6	3.9	7	4.6	6	3.9
24 May 1979	131	4.6	3	2.3	2	1.5	1	0.8
19 Jul. 1979	214	13.6	15	7.0	13	6.1	6	2.8
26 Jul. 1979	38	0.0	0	0.0	0	0.0	0	0.0
11 Oct. 1979	312	22.1	44	14.1	32	10.3	19	6.1

**The sum of the row percentages do not always equal the percentage of isolates producing coliform-inhibitory substances because of BLS cross-specificity.*

Table 4. *Analysis of variance: size of the zone of inhibition for* E. coli, Klebsiella *spp., and* E. aerogenes *by site, date, and genus*

	Diameter of zone of inhibition					
	Site		Date		Genus	
Coliform	*F*	*P*	*F*	*P*	*F*	*P*
E. coli	20.7	<0.001	7.5	<0.001	5.9	<0.001
Klebsiella *sp.*	6.7	<0.001	7.5	<0.001	5.0	<0.001
E. aerogenes	8.6	<0.001	2.1	<0.05	4.6	<0.001

Table 5. *Chi-square analysis: frequency of coliform-specific BLS producers by site, date, and bacterial genus*

	Site			Date			Genus		
Coliform	X^2	*P*	*V*	X^2	*P*	*V*	X^2	*P*	*V*
E. coli	118.9	0.001	0.24	90.5	0.001	0.21	91.7	0.001	0.21
Klebsiella *sp.*	79.8	0.001	0.16	55.4	0.001	0.16	69.3	0.001	0.18
E. aerogenes	43.7	0.001	0.15	27.1	0.001	0.12	53.3	0.001	0.16

Table 6. *Numerical breakdown of bacterial genera producing BLS for* Enterobacter, Aerogenes, Klebsiella *spp., or E. coli*

	Inhibition of E. aerogenes			*Inhibition of* Klebsiella *sp.*			*Inhibition of* E. coli		
Group	*Total no. tested*	*No. inhibiting*	*%*	*Total no. tested*	*No. inhibiting*	*%*	*Total no. tested*	*No. inhibiting*	*%*
Actinomycetes	44	1	2.2	44	2	4.5	44	1	2.2
Acinetobacter *spp.*	178	0	0.0	178	9	4.5	178	8	4.5
Pseudomonas *spp.*	18	0	0.0	18	0	0.0	18	2	11.1
Pasteurella *spp.*	16	0	0.0	16	4	25.0	16	5	31.3
Pseudomonas/Alcaligenes *spp.*	268	4	1.5	267	2	0.7	267	5	1.9
Flavobacterium/Moraxella *spp.*	351	28	8.0	353	37	10.5	353	53	15.0
Klebsiella *spp.*	114	4	3.5	114	8	7.0	114	3	2.6
Serratia *spp.*	14	0	0.0	14	0	0.0	14	2	14.3
Enterobacter *spp.*	7	2	28.6	7	2	28.5	7	2	28.5
*Unknown**	64	5	7.8	64	2	3.1	64	6	9.4
Unidentified†	70	1	1.4	70	1	1.4	70	0	0.0
Total	1,144	45	3.9	1,145	66	5.8	1,145	87	7.6

**Unidentifiable by Lassen method.*
†Not yet identified.

Figure 9. *Scanning electron micrograph showing a bacterially-colonized particle recovered from a water distribution system. Rod-shaped bacterial cells are visible. Note that the majority of the cells appear to be superficially attached without extensive glycocalyx formation. Bar = 5.0 µm.*

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***Donald Reasoner** is the EPA Project Officer (see below).*
The complete report, entitled "Assessment and Implications of Bacterial Regrowth in Water Distribution Systems," (Order No. PB 82-249 368; Cost: $21.00, subject to change) will be available only from:
National Technical Information Service
5285 Port Royal Road
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Telephone: 703-487-4650
The EPA Project Officer can be contacted at:
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